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Hazard Evaluation Division Standard Evaluation Procedure Fish Early Life-Stage Test

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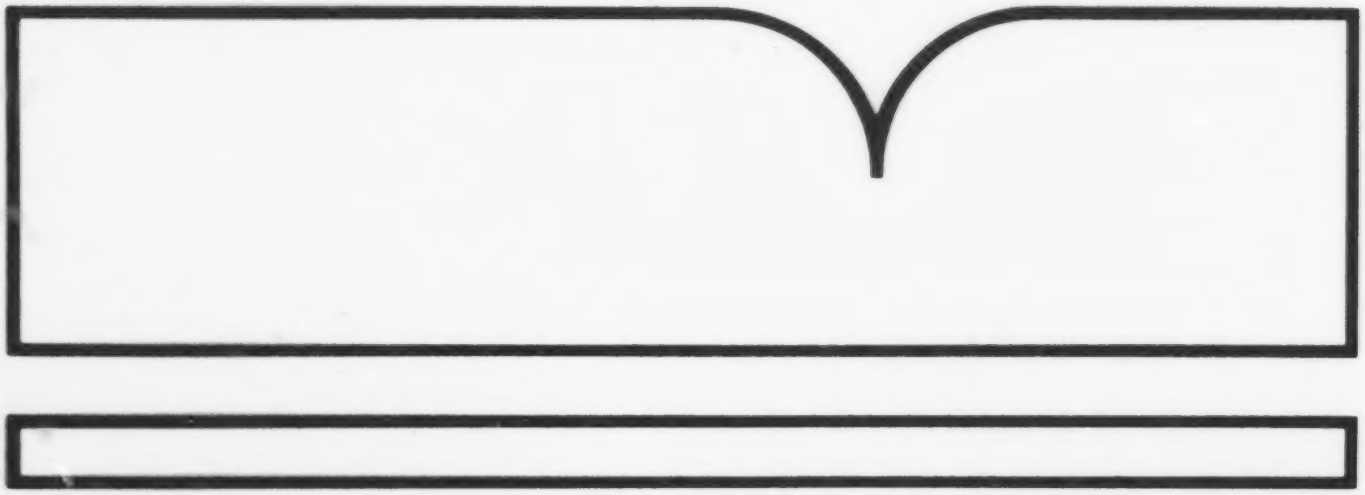

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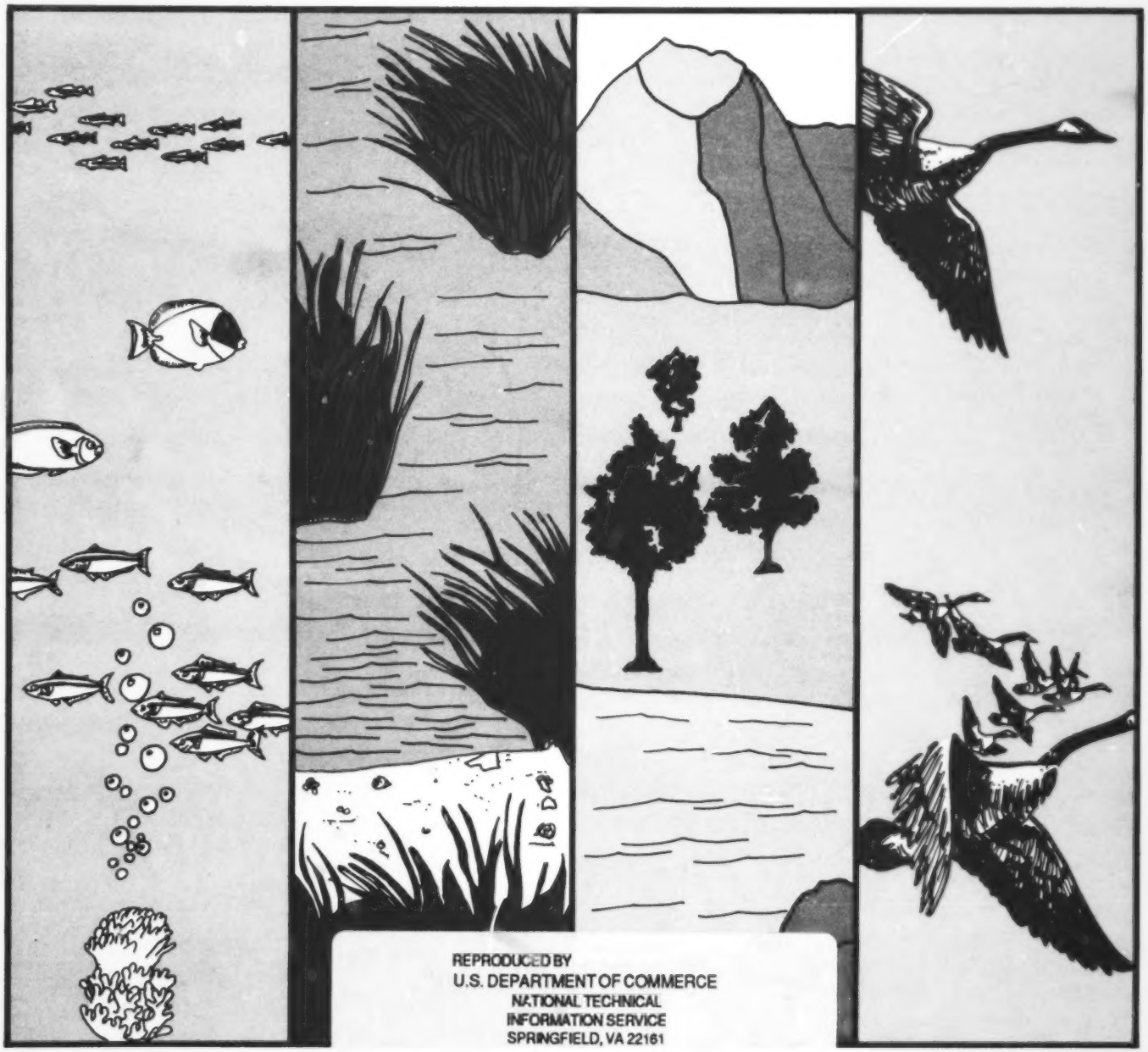

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The Standard Evaluation Procedure (SEP) for fish early life-stage testing is a document primarily intended as guidance for Agency reviewers and the regulated industry. As a result of application for registration, pesticide producers are required by 40 CFR Part 158.145 of the regulations to provide fish life-stage testing. It is intended to be used in conjunction with Subdivision E of the Pesticide Assessment Guidelines: Hazard Evaluation - Wildlife and Aquatic Organisms.

The SEP was compiled from ideas and methods that are being used by EPA laboratories, private laboratories and the ASTM (American Society for Testing and Materials) in developing a testing procedure that will 1) establish chronic toxicity levels of the toxicant to fish; 2) compare toxicity information with measured or estimated toxicant residues in an aquatic environment; 3) indicated the need for further laboratory testing or field testing.

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HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

FISH EARLY LIFE-STAGE TEST

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STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.

John W. Melone, Director
Hazard Evaluation Division

TABLE OF CONTENTS

	Page
I. INTRODUCTION	
A. When Required	1
B. Purpose	1
C. Test Material	2
D. Acceptable Protocols	2
II. MATERIALS, METHODS, AND REPORTING REQUIREMENTS	
A. Biological System	2
1. Acceptable Species	2
2. Source	2
3. Eggs from Adult Fish	3
4. Embryo Exposure (Test Begins)	3
5. Post Hatch, Larval Fish	4
6. Controls	5
7. Data Endpoints	5
B. Physical System	5
1. Test Water	5
a. Saltwater Fish	5
b. Freshwater Fish	6
2. Temperature	6
3. Photoperiod	6
4. Dosing Apparatus	6
5. Toxicant Mixing	6
6. Test Vessels	6
7. Embryo Cups	6
8. Flow Rate	7
9. Aeration	7
C. Chemical System	7
1. Concentrations	7
2. Measurement of Other Variables	7
3. Solvents	7
D. Calculations	8
III. REVIEWER'S EVALUATION	
A. Verification of Statistical Analysis	8
B. Conclusions	8
1. Categorization of Results	8
2. Rationale	9
3. Reparability	9
4. Descriptive Conclusions	9
REFERENCES	10

FISH EARLY LIFE-STAGE

I. INTRODUCTION

A. When Required

The fish early life-stage test[1/] is required to support an end-use product is intended to be applied directly to water or is expected to transport to water from the intended use site, and when any one of the following conditions apply:

- If the pesticide is intended for use such that its presence in water is likely to be continuous or recurrent regardless of toxicity, as revealed by studies required by 40 CFR §158.130

- If any LC_{50} or EC_{50} value determined in the testing required by 40 CFR §158.145 [§§ 72-1, -2, or -3] is less than 1 mg/l;

- If the estimated environmental concentration in water is equal to or greater than 0.01 of any EC_{50} or LC_{50} determined in acute testing required by 40 CFR §158.145; or

- If the actual or estimated environmental concentration in water resulting from use is less than 0.01 of any EC_{50} or LC_{50} determined in testing required by 40 CFR §158.145 and any of the following conditions exists:

  - Studies of other organisms indicate the reproductive physiology of fish and/or invertebrates may be affected;

  - Physicochemical properties indicate cumulative effects; or

  - The pesticide is persistent in water (e.g., half-life in water greater than four days).

B. Purpose

- To establish chronic toxicity levels of the active ingredient to fish;

1/ In cases where risk criteria for both fish and invertebrates are exceeded, the more sensitive organism must be tested in a fish early life-stage or invertebrate life cycle study. Both studies may, however, may be required to complete a risk assessment.

- To compare toxicity information with measured or estimated pesticide residues in an aquatic environment in order to assess potential impact to fish;
- To provide support for precautionary label statements; and
- To indicate the need for further laboratory testing or field testing.

C. Test Material

Testing must be conducted with the technical grade of the active ingredient (a.i.). If more than one active ingredient constitutes a technical product, the technical grade of each active ingredient must be tested separately.

D. Acceptable Protocols

The Ecological Effects Branch (EEB) does not endorse any one protocol. It is sometimes necessary and desirable to alter the procedures presented in published protocols to meet the needs of the chemical or test organisms used. However, EEB does recommend some protocols as guidance for performing a fish early life-stage toxicity test. These protocols include:

American Public Health Association, American Water Works Association and Water Pollution Control Federation (1985) Standard Methods for the Examination of Water and Wastewater. Sixteenth Edition. Publication Office: American Public Health Association, 1015 18th Street NW, Washington, DC 20036. 854 pp.

Goodman, L.R. (1985) Comparative Toxicological Relationship Demonstrated in Early Life. Stage Tests with Marine Fish. Environ. Res. Lab., Gulf Breeze, FL. EPA/600/X85/135.

Middaugh, D.P., M.J. Hemmer, and L.R. Goodman. 1987. Methods for Spawning, Culturing and Conducting Toxicity Tests with Early Life Stages of Four Atherinid Fishes: The inland silverside, Menidia beryllina, Atlantic silverside, M. menidia, tidewater silverside, M. peninsulae, and California grunion, Leuresthes tenuis. Office of Research and Development, U.S. Environmental Protection Agency, Environmental Research Laboratory at Gulf Breeze Florida, EPA/600/8-87/004 (Janurary, 1987), 56 pp.

II. MATERIALS, METHODS, AND REPORTING REQUIREMENTS

A. Biological System

1. Acceptable Species

The selected species should have a demonstrated sensitivity to known toxicants. If possible, they should be species that occur in the area of exposure or be related to exposed species.

The acceptable freshwater species,(1, 2) are rainbow trout (Salmo gairdneri), brook trout (Salvelinus fontinalis), coho salmon (Oncorhynchus kisutch), Chinook (O. tshawytscha), bluegill (Lepomis macrochirus), brown trout (S. trutta), lake trout (S. namaycush), northern pike (Esox lucius), fathead minnow (Pimephales promelas), white sucker (Catostomus commersuni), and channel catfish (Ictalurus punctatus). The silverside species (Menidia menidia, menidia beryllina, and Menidia peninsulae) and the sheepshead minnow (Cyprinodon variegatus) are acceptable estuarine species.(1, 2)

2. Source

Gametes used for conducting a fish early life-stage test may be obtained: 1) directly from hatcheries or commercial sources; 2) from wild populations of adult fish collected in the field; or 3) from brood fish cultured in the laboratory. Whenever salmon or trout are used, they should be obtained from a hatchery that has been certified disease-free.

3. Eggs from Adult Fish

Eggs can be obtained either by stripping ripe females or by collecting eggs deposited directly on substrata. Manual removal is usually conducted on salmon, pike, trout, bluegill, and silversides. This procedure is usually preceded by killing or by anesthetizing ripe females in MS-222 (≈ 100 ppm) or quinaldine (≈ 10 ppm). Eggs are forced from the vent by manual or air pressure techniques. If fish are sacrificed, an incision is made along the median ventral line from the vent to the pectoral fins of ripe females. Care should be taken to keep eggs free from mucous and blood. Eggs still adhering to the ovaries are not taken. Eggs from at least three females should be fertilized with sperm from at least three males. Male specimens (e.g., salmon, trout) anesthesized with ≈ 100 ppm of MS-222 can be collected by gently pressing the sides of the abdomen.(1) Sperm collection from pike is best accomplished by sacrificing the fish.(1) The fish are killed and the testes removed through an incision in the abdomen. Testes are placed in clean cheesecloth and squeezed to extrude milt.(1)

Fertilized eggs may be shipped but can be damaged by rough handling. Eggs should be water hardened for one-hour prior to shipping and kept cool (< 10°C).(1) Preferably eggs and sperm should be shipped separately with plenty of ice and a blanket of pure oxygen on the sperm. Unfertilized eggs and semen can be transported for a period of 24 hours after stripping if they are kept in an air-tight container.(1) Using a 0.75 percent saline solution when mixing eggs and semen may facilitate fertilization.

Eggs can be obtained from channel catfish, fathead minnow, sheepshead minnow, and bluegill by facilitating natural spawning either in the laboratory or a brood pond.(1, 3, 4, 5, 6)

4. Embryo Exposure (Test Begins)

Embryos can be either fertilized prior to toxicant exposure or fertilized in the test solution. Verification of the precise embryonic stage at the beginning of the exposure should be attempted if possible.

Eggs used to initiate the study should be at the eyed stage, and selected from a group of which 70% are fertilized.

A minimum of 20 embryos are randomly selected per replicate cup with four replicates per concentration (80 embryos total).(1) Cups containing embryos are placed into the exposure chambers. Water may flow directly over the embryos in the cup or the cups may be oscillated in the test solution by means of rocker arm apparatus driven by a low speed electric motor.

Embryos should be 2 to 24 hours old at the beginning of the test. Twenty-four hours after being placed in the incubation cups they should be counted and examined for dead or heavily fungused individuals, which should be discarded without disturbing the viable embryos.(2) This counting and examination is repeated on a daily basis. The range of time-to-hatch in each cup is species specific as noted in table 1.

Table 1. Average Time-to-Hatch for Several Species of Fish, Relative to Temperature(1)

Species	Temperature	Days-to-Hatch
Rainbow trout (Salmo gairdneri)	10 °C	31
Brook trout (Salvelinus fontinalis)	10 °C	44
Coho salmon (Oncorhynchus kisutch)	10 °C	55
Brown trout (Salvelinus trutta)	10 °C	41
Lake trout (Salvelinus namaycush)	7 °C	72
Chinook (Oncorhynchus tshawytscha)	10 °C	56
Northern pike (Esox lucius)	15 °C	6
Bluegill (Lepomis macrochirus)	28 °C	6
Fathead minnow (Pimephales promelas)	25 °C	5
Channel catfish (Ictaluras punctatus)	26 °C	6-7
Sheepshead (Cyprinodon variegatus)	30 °C	4
	25 °C	7
Silverside (Menidia menidia)	25 °C	8

5. Post Hatch, Larval Fish

When hatching is about 90 percent completed or 48 hours after first hatch, live young fish should be counted.(1) All of the normal and abnormal live fish should be released into the test chambers.(1) Fish numbers can be thinned to at least 30 per treatment.(1) A test should be terminated if the average percent of embryos (based on the number of embryos after thinning) that produce live fry for release into the test chambers in any control treatment is less than 50 percent or if the percent hatch in any control embryo cup is more than 1.6 times that in another control embryo cup.(1, 3)

Test fish over two days old (post hatch swim-ups) must be fed live newly hatched brine shrimp.

Fish should be fed at least twice daily. Time between feedings will be species specific, and must be based on a reliable hatchery feeding schedule. Control and treatment fish must receive equal amounts of food if growth is to be a meaningful endpoint.

Dead fish should be removed and recorded when observed. At a minimum, the live fish should be counted (including those which are lethargic or grossly abnormal in either swimming behavior or physical appearance) 11, 18, 25, and 32 days after hatching.(1)

Fish should not be fed for at least 24 hours prior to termination on day 32. At termination, all live fish should be weighed (wet, blotted dry).

6. Controls

A test is not acceptable if the average survival of the controls at the end of the test is less than 80 percent or if survival in any control chamber is less than 70 percent.(1) The relative standard deviation (RSD = 100 x standard deviation divided by mean) of weights of the fish that were alive at the end of the test in any control test chamber must not be greater than 40 percent.(1)

A negative control (no toxicant or carrier) and a carrier control (when applicable) are required. Regardless of the carrier used, the carrier concentration should be equal in each exposure concentration and carrier control. If they are not, the carrier concentration in the control (carrier) must be at least as high as that in any toxicant test chamber.

7. Data Endpoints

A record of the results of an acceptable test must include the number of embryos hatched, time to hatch, mortality of embryos, larvae, and juveniles, time to swim-up, measurement of growth, incidence of pathological or histological effects, and observations of other effects or clinical signs in each treatment. Endpoints defined in terms of statistically significant differences and biologically significant differences are based on contingency table, or other hypothesis testing procedures and regression analysis, concentration-effect curve analysis, and other estimation procedures.(1) Tests for chamber to chamber heterogeneity within treatments are generally based on analysis of variance or contingency table procedures.(1)

B. Physical System

1. Test Water

a. Saltwater Fish

1) Test water may be natural (sterilized and filtered to remove particles 15 mierons and larger) or a commercial mixture (provided that there are no adverse affects to test organisms or alterations in test material toxicity); 2) Natural seawater is considered to be of constant quality if the weekly range of salinity is less than six percent, and if monthly pH range is less than 0.8 of a pH unit; 3) Salinity should be $\geq$ 15 parts per thousand; 4) Water must be free of pollutants.(7) Use of ultraviolet light irradiation is recommended to sterilize the test water.

b. Freshwater Fish

1) Test water can be supplied from a well or spring provided that the source is not polluted; 2) Water should be sterilized with ultraviolet irridiation and tested for pesticides, heavy metals, and other possible contaminants; 3) Hardness of 40 to 48 mg/L as $CaCO_3$ and pH of 7.2 to 7.6 is recommended; 4) Reconstituted water can be used. Detailed descriptions of acceptable procedures for preparing diluent are found in the protocols by the American Society of Testing Materials (1980).(2)

2. Temperature

Test temperature depends upon the test species and should not deviate by more than 2°C from the appropriate temperature (refer to section A, 4).

3. Photoperiod

A photoperiod of 16L/8D can be used with a light intensity of 400 to 800 Lux at the surface of the test solution. However, in general salmonid eggs should be incubated under dim lighting (< 20 ft-candles) or total darkness.(1)

4. Dosing Apparatus

Intermittent-flow proportional diluters as described by Mount and Brungs (8) or continuous-flow serial diluters, as described by Garton (7) should be employed. A minimum of five toxicant concentrations with a dilution factor not greater than 0.50 and controls should be used.

5. Toxicant Mixing

A mixing chamber is recommended to assure adequate mixing of test material. Aeration should not be used for mixing. Separate flow splitter delivery tubes should run from this container to each replicate larval tank.(3) Depending upon the apparatus used, a mixing chamber may not be required, but it must be demonstrated that the test solution is completely mixed before introduction into the test system. Flow splitting accuracy must be within 10 percent and should be checked periodically for accurate distribution of test water to each tank.(1)

6. Test Vessels

All test tanks should be of either all glass or glass with a stainless steel frame. Exposure vessels will vary in size according to the species under test. Generally, it is desirable to have a depth of water of at least 15 to 30 cm.(1)

7. Embryo Cups

Embryo incubation cups should be made from 120 mL glass jars with the bottoms replaced with 40 mesh stainless steel or nylon screen. Cups can be oscillated vertically (2.5 to 4.0 cm) in the test water (rocker arm apparatus, 2 rpm motor) or placed in separate chambers with self-starting siphons. Both methods should insure adequate exchange of water and test material.

8. Flow Rate

Flow rates to larval cups should provide 90 percent replacement in 8 to 12 hours.(3) Flow rate must be capable of maintaining dissolved oxygen at above 75 percent of saturation and maintain the toxicant level (concentration cannot drop below 20 percent with fish in the tank).

9. Aeration

Dilution water should be aerated vigorously insuring that dissolved oxygen concentration will be at or near 90 to 100 percent saturation. Test tanks and embryo cups should not be aerated.

C. Chemical System

1. Concentrations

A minimum of five concentrations of toxicant and a control, (all replicated) are used in this chronic test. A solvent control is added if a solvent is utilized. At a minimum, the concentration of toxicant must be measured in one tank at each toxicant level every week. Water samples should be taken about midway between top and bottom and the sides of the tank. One concentration selected must adversely affect a life-stage and one concentration must not affect any life-stage.

2. Measurement of Other Variables

Dissolved oxygen must be measured at each concentration at least once a week. Freshwater parameters in a control and one concentration must be analyzed once a week. These parameters should include pH, alkalinity, hardness, and conductance. Natural seawater must maintain a constant salinity and not fluctuate more than six percent weekly or a monthly pH range of less than 0.8 of a pH unit.

3. Solvents

If solvents other than water are necessary, they should be used sparingly and not to exceed 0.1 mL/L in a flow-through system. The following solvents are acceptable:(2)

dimethylformamide
triethylene glycol
methanol
acetone
ethanol

The development of chemical saturators for use with hydrophobic chemicals may be used with most test chemicals.(6, 9)

D. Calculations

Data from these toxicity studies are of two types, continuous (i.e., length, weight) and discrete (i.e., number of fish hatching or surviving). In general, continuous data should be analyzed with the appropriate analysis of variance (ANOVA) technique followed by an appropriate multiple comparison test. Dichotomous data should be analyzed using some form of a 2 x 2 contingency table.

As a part of the ANOVA, it is desirable to plot the residuals versus concentration and determine whether there have been any obvious violations of homoscedasticity on the assumption of normality. All test results must be accompanied by copies of the original (raw) data for the reviewer's evaluation. Transcripts of the original raw data may be submitted if they provide all of the information available in the original, including comments or notes of the investigator.

III. REVIEWER'S EVALUATION

The reviewer should identify each aspect of the reported procedures and determine if there is any inconsistency with recommended methodologies. The number of deviations and their severity will determine the validity of the study and the interpretation of the results.

A. Verification of Statistical Analysis

Reviewer should ensure that a maximum allowable toxic concentration (MATC) has been properly derived by recalculating the reported results. If the recalculated results differ substantially from the submitted results, the reviewer should note this and attempt to explain the differences.

B. Conclusions

1. Categorization of Results

The significance of inconsistencies in the test procedures must be determined by the reviewer so that the results of the test can be categorized as to whether they fulfill Part 158 regulations and are useful in performing a risk assessment. Categories are described as:

° Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

° Supplemental: Studies in this category are scientifically sound; however, they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements; however, the information may be useful in a risk assessment.

Some of the conditions that may place a study in a supplemental category include:

- Unacceptable test species;
- Inappropriate test material; or
- Deviations from recommended test solution characteristics (variations in DO, temperature, hardness, and pH can affect toxicological response).

° Invalid: These studies provide no useful information. They may be scientifically unsound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment.

Fxamples of studies placed in this category commonly include those where the test system was aerated, test vessels were constructed from materials other than glass, or there were problems of solubility or volatility of the test material. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that test organisms were actually exposed to nominally designated concentrations.

A study where the test material was not properly identified can also be invalidated.

2. Rationale

Identify what makes the study supplemental or invalid. While all deviations from recommended protocol should be noted, the reviewer is expected to exercise judgment in the area of study categorization.

3. Reparability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data about the study.

4. Descriptive Conclusions

The reviewer should indicate what the results were and how much information can be drawn from them. These results are useful in a risk assessment.

REFERENCES

(1) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race street, Philadelphia, PA 19103.

(2) Benoit, D.A. (1981) User's Guide for Conducting Life-Cycle Chronic Toxicity Tests with Fathead Minnows (Pimephales promelas). Environ. Res. Lab.-Duluth, Duluth, MN. EPA-600/8-81-011.

(3) Hansen, D.J.; Parrish, P.R.; Schimmel, S.C.; Goodman, L.R. (1978) Toxicity Test Using Sheepshead Minnows (Cyprinodon variegatus). Bioassay Procedures for the Ocean Disposal Permit Program. EPA-600/9-78-010.

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(6) Garton, R.R. (1980) A Simple Continuous-Flow Toxicant Delivery System. Water Res. 14:227-230 pp.

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